



TITLE:

α' Subunit of soybean β -conglycinin forms complex with rice glutelin via a disulphide bond in transgenic rice seeds

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1 Title: α' subunit of soybean β -conglycinin forms complex with rice glutelin via a disulfide bond in
2 transgenic rice seeds

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25

1 **Abstract**

2 We expressed the α' and β subunits of soybean β -conglycinin in rice seeds to improve the
3 nutritional and physiological properties of rice as a food. The α' subunit accumulated in rice seeds at a
4 higher level than the β subunit, but no detectable difference in mRNA transcription level between subunits
5 was observed. Sequential extraction results indicate that the α' subunit formed one or more disulfide bonds
6 with glutelin. Electron microscopic analysis showed that the α' subunit and the β subunit were transported
7 to PB-II in analogy with glutelin. In mature transgenic seeds, the β subunit accumulated in low electron
8 density regions in the periphery of PB-II, whereas the α' subunit accumulated together with glutelin in
9 high-density regions of the periphery. The subcellular localization of mutated α' subunits lacking one
10 cysteine residue in the N-terminal mature region ($\alpha'\Delta\text{Cys1}$) or five cysteine residues in the pro and
11 N-terminal mature regions ($\alpha'\Delta\text{Cys5}$) were also examined. Low-density regions were formed in PB-II in
12 mature seeds of transgenic rice expressing $\alpha'\Delta\text{Cys5}$ and $\alpha'\Delta\text{Cys1}$. $\alpha'\Delta\text{Cys5}$ was localized only in the
13 low-density regions, whereas $\alpha'\Delta\text{Cys1}$ was found in both low- and high-density regions. These results
14 suggest that the α' subunit could make complex via one or more disulfide bonds with glutelin and
15 accumulated together in PB-II of transgenic rice seeds.

16

1 Introduction

2 Rice storage proteins are composed of glutelin (acid/alkaline-soluble), prolamin (alcohol-soluble),
3 globulin (saline-soluble), and albumin (water-soluble). Glutelins, account for about 80 % of the total
4 proteins in rice seeds (Ogawa et al., 1987; Li and Okita, 1993; Cagampang et al., 1966). They are
5 synthesized as a precursor which is further processed proteolytically into acidic and basic chains connected
6 with a disulfide bridge like 11S globulin. Rice proteins are rich in sulfur-containing amino acids, but are
7 deficient in lysine. In contrast, β -conglycinin (7S globulin of soybean) and glycinin (11S globulin of
8 soybean) are rich in lysine but are poor in sulfur-containing amino acids (Utsumi, 1992). Moreover, their
9 physiological benefits to humans have been established. β -conglycinin lowers plasma cholesterol and
10 triglyceride levels in human (Sirtori et al., 1995; Aoyama et al., 2001). The α' subunit of β -conglycinin has
11 been reported to have LDL-cholesterol-lowering activity (Sirtori and Lovati, 2001) and
12 phagocytosis-stimulating activity (Tsuruki et al., 2003). Therefore, an accumulation of β -conglycinin in
13 rice seeds could lead to the development of a product with high nutritional value, several important
14 physiological activities and useful physicochemical properties.

15 β -Conglycinin has a trimeric structure similar to other 7S globulins. The α and α' subunits
16 contain an N-terminal extension in addition to a core region common to all the subunits. The β subunit
17 consists of only the core domain (Maruyama et al., 1998). The extension regions of the α and α' subunits

1 probably protrude from the molecular surface of the core domains and have a minor role in proper folding
2 and assembly (Maruyama et al., 2001). The α/α' subunits and the β subunit are synthesized on polysomes
3 as prepro- and pre-forms, respectively. The signal peptides are cotranslationally removed, the polypeptides
4 are N-glycosylated with high-mannose glycans and assemble into trimers in the ER (Yamauchi and
5 Yamagishi, 1979; Utsumi, 1992). The resultant α/α' subunits and the β subunit are in pro-form and mature
6 form, respectively. Each trimer is transported from the ER to the protein storage vacuoles through the
7 Golgi apparatus (Mori et al., 2004). The pro regions of the α and α' subunits are proteolytically processed
8 to give their mature forms. Both the α and α' subunits contain four cysteine residues (Cys) in their pro
9 regions and one Cys in the mature extension region (Cys13).

10 In rice seeds, there are two types of protein bodies, PB-I and PB-II. PB-I, derived directly from
11 the ER, contains mainly prolamin (Tanaka et al., 1980; Yamagata et al., 1986). Binding protein (BiP)
12 facilitates folding and assembly of prolamin polypeptides (Li et al., 1993). PB-II, derived from the
13 vacuoles, have non-spherical structure and primarily contain glutelin and globulin (Tanaka et al., 1980).
14 Both proteins are synthesized on the rER, assembled in the ER, transported to the Golgi apparatus, and
15 sorted to the vacuole. Both glutelin and globulin are transported from the Golgi apparatus to storage
16 vacuoles by the so-called dense vesicles (Krishnan et al., 1986, 1992). However, glutelin and globulin
17 accumulate in different regions of PB-II (Krishnan and White, 1995). Globulin accumulates in the

1 periphery region of the PB-II (Krishnan et al., 1992; Krishnan and White, 1995).

2 To develop rice having a high nutritional value and physiological function, we introduced and
3 expressed the α' and β subunits of soybean β -conglycinin in rice seeds. Further, we analyzed the traffic
4 and accumulation behavior of the α' and β subunits in rice seeds. Our results indicate that the α' subunit
5 accumulates at a higher level than the β subunit and that the α' subunit forms a complex via one or more
6 disulfide bonds with glutelin in transgenic rice seeds.

7

8 **Materials and methods**

9 **Construction of binary vectors and transformation**

10 cDNAs encoding the α' and β subunits were modified to remove a *Sac* I cleavage site in the
11 coding sequence while retaining the actual amino acid sequence, and to introduce the *Sac* I site at the
12 downstream of the stop codon by PCR using the following pairs of oligonucleotide primers : 5'-
13 gatatgaacgaggggctcttttctgccca -3' and 5'- cacaacactgaggaagacatccaagtcc -3' for α' ;
14 5'-ggatatcaacgaaggcgctcttcttacc-3' and 5'-acagaactgaggaagatatccaagtcccg-3' for β ;
15 5'-atgatgagagcgcggttccattac-3' and 5'-catcatgcgagctctcagtaaaaagccctc-3' for α' ;
16 5'-atgatgagagtgcggttctcttg-3' and 5'-catcatgcgagctctcagtagagagcacctaag-3' for β (underlines indicate *Sac* I
17 cleavage site). The resulting modified α' and β cDNAs were digested by *Sac* I. A *Sal* I cleavage site was

1 introduced at 5' ends of glutelin promoters *GluB-1* and *GluB-2*, which direct the expression at the
2 periphery of the endosperm (Takaiwa et al., 1996, Wu et al., 1998), by PCR using the following primers:
3 5'-agctattgttacttgcttatggaagc-3' and 5'-acttcacaaagtagtagtcaacc-3' for *GluB-1* promoter;
4 5'-agctattagcagttgctaataaggaaac-3' and 5'-gaggaatagagataaggttgaggag-3' for *GluB-2* promoter (underlines
5 indicate *Sal* I cleavage site). The modified promoter regions were digested by *Sal* I. The 2.3 kb *GluB-1* and
6 2.4kb *GluB-2* promoter regions were ligated with the α' and β cDNAs between *Sal* I and *Sac* I cleavage
7 sites of pBluescript SK (Stratagene, La Jolla, CA, USA), respectively. The DNA sequences of promoter
8 and cDNA regions inserted into pBluescript SK were confirmed by DNA sequencing using the following
9 primers: 5'-ccaaggaaaagctcgtatttagtgag-3', 5'-gacgcgggagccgtcctaggtgcaccgg-3',
10 5'-tggaataattacatacaccaatatg-3' for *GluB-1*; 5'-atgatgagagcgcggttccc-3', 5'-cgaagacataagaataagaaccc-3',
11 5'-gtttcttctatcttagcac-3', 5'-aaacctttcaacttgagaagcc-3' for α' ; 5'-attaccatccccataaccagaaactc-3',
12 5'-aagttgtgagtgtagtca-3', 5'-acacaacaatttgatgtttccag-3', 5'-ggcaagacacatactaaaagtatgg-3' for *GluB-2*;
13 5'-atgatgagagtgcggtttcc-3', 5'-gccatacccgtaacaaacctggca-3', 5'-ggatatcaacgaaggagctcttcttacc-3',
14 5'-tggtctgcacaagatgttgagagg-3' for β . For α' Δ cys1, the codon of Cys13 was replaced with that of Ser in
15 the pBluescript SK using the following primers: 5'-gtggaggaagaagaagaagcgaagaagg-3' and
16 5'-cttaaggaggttgcaacgagcgtgg-3'. For α' Δ cys5, the pro-region of α' was deleted and the codon of Cys13
17 was replaced with that of Ser in the pBluescript SK using the following primers:

1 5'-gtggaggaagaagaagaagcgaagaagg-3' and 5'-aatgccaaatgagacagaaactgatgc-3'. The DNA region coding
2 *GluB-1- α '*, - α' Δ Cys1 and - α' Δ Cys5 in pBluescript SK was cleaved out by *Sac* I and *Sal* I, and these DNA
3 fragments were inserted into a binary vector pGTV-HPT (Becker et al., 1992), where *Sac* I linker was
4 introduced, to construct pGTV-HPT/ α' , pGTV-HPT/ α' Δ Cys1 and pGTV-HPT/ α' Δ Cys5, respectively.
5 *GluB-2* promoter and β cDNA were digested by *Sac* I, because there is a *Sac* I cleavage (-1759) site at
6 upstream of *GluB-2* promoter, and the resulting DNA fragment was inserted into pGTV-HPT cleaved by
7 *Sac* I to give pGTV-HPT/ β . The direction of the insert was checked by DNA sequencing using the
8 following primer: 5'-tgggtctgcacaagatgtga-3'.

9 pGTV-HPT/ α' , pGTV-HPT/ α' Δ Cys1, pGTV-HPT/ α' Δ Cys5 and pGTV-HPT/ β were introduced
10 into *Agrobacterium tumefaciens* (EHA105) (Hood et al., 1993) by electroporation as described previously
11 (Goto et al., 1999). Rice (cv. Kitaake) calli were infected with these *Agrobacterium*. Hygromycin-resistant
12 calli were selected and plants were regenerated in the presence of 50 mg/l hygromycin.

13 Antibodies

14 Antisera against α' subunit (Nishizawa et al., 2003) and β subunit (Maruyama et al., 1998) of
15 β -conglycinin, rice proglutelin (Katsube et al., 1999), 10kDa and 16kDa prolamins (will be described
16 elsewhere) and soybean BiP (Mori et al., 2004) raised in rabbit were used.

17 Quantification of expression levels of β -conglycinin in the rice seeds

1 Primary transgenic rice plant seeds (T₁) were dehulled, and ground separately with a mortar and
2 pestle. Proteins were extracted with 45 mM Tris-HCl (pH 6.8) containing 2 % (w/v) SDS, 30 % glycerol,
3 and 0.1 M ME (2-mercaptoethanol). Aliquots (1μg) of protein were then spotted on a nitrocellulose
4 membrane and β-conglycinin was detected immunologically with either anti-α' or anti-β sera (Nishizawa
5 et al., 2003; Maruyama et al., 1998). The accumulation levels of α' and β subunits were estimated by
6 comparing the densitometric signals obtained from the extracts prepared from the transgenic plants with
7 those obtained from the extract prepared from the non-transgenic plant containing a known amount of
8 purified α' or β from soybean seeds. Six individual seeds were analyzed from individual transgenic plants
9 and the maximum accumulation level within six seeds was used to compare expression levels between
10 constructs.

11 **SDS-PAGE and Western blotting**

12 SDS-PAGE was done using 11% polyacrylamide gel. Proteins were stained with coomassie
13 brilliant blue R-250. Western blotting was done after SDS-PAGE using 11% polyacrylamide gel. The
14 separated proteins on gels were transferred electrophoretically to nitrocellulose membrane (0.45 μm;
15 Schleicher and Schuell Inc., Dassel, Germany) and recombinant proteins were detected with anti-α',
16 and/or anti-β sera followed by a goat anti-rabbit IgG-alkaline phosphatase conjugate (Promega, Madison,
17 WI, USA).

1 **2D SDS-PAGE**

2 Two-dimensional (without/with ME) SDS-PAGE was done using 7.5 % gels. Sample lanes from
3 the first dimension (without ME) were cut off from the gel and incubated with SDS-PAGE running buffer
4 containing 0.1 M ME for 1 hr at room temperature, subjected to SDS-PAGE in the second dimension (with
5 ME) and analyzed by Western blotting.

6 **Comparison of transcription levels of α' and β mRNAs in rice seeds**

7 Total RNA was extracted from immature homozygous T₂ seeds (about 15 days after pollination).
8 Six seeds were ground with a mortar and pestle in phenol solution (phenol: chloroform: isoamyl alcohol =
9 25:24:1). After addition of TE buffer (0.1 M Tris-HCl (pH 9.0), 1 % (w/v) SDS, 0.1 M NaCl, 5 mM
10 EDTA), total RNA was purified by lithium chloride precipitation and DNase digestion (Goto et al., 1999).

11 Transcription levels of α' and β mRNAs were measured by real-time PCR. This assay was
12 carried out in the ABI-PRISM 7000 (Applied Biosystems) using the Taqman system (Applied Biosystems)
13 in a final volume of 50 μ l. The reaction mixture including 5.5 mM MgCl₂, 0.3 mM each of dATP, dCTP,
14 and dGTP, 0.8 mM dUTP, 0.2 μ M forward and reverse primers (5'-ttgtttgagattaccccagagaaaa-3' and
15 5'-cctcggtcatatccacaactga-3' for α' subunit, 5'-gactaccggattgtccagtttca-3' and 5'-aatcggcgctcagcatggt-3'
16 for β subunit, and 5'-cgaggcgagtcgaaga-3' and 5'-cccagttgctgacgatacca-3' for rice actin gene (RAC1) as
17 an internal standard), 12.5 U reverse transcriptase, 20 U RNase inhibitor, 1.25 U DNA polymerase, and 0.1

1 μM Taqman probe. The probes for α' and β subunits were 5'-FAM-ccctcagcttcgggacttgatgtct-TAMRA-3'

2 and 5' - FAM-tcaaaacccaacacaatccttctcccc-TAMRA-3', respectively. The probe for RAc1 was

3 5'-VIC-tatcttgaccctcaagtaccccatcgag-TAMRA-3'. These primers and probes were designed by Primer

4 Express ver 2.0 (Applied Biosystems). Conditions for amplification were 30 min at 48 °C, 10 min 95 °C,

5 and 40 cycles of 15 sec at 95 °C and 1min at 60 °C. Results were analyzed using a sequence detection

6 system (ABI prism 7000 SDS system) provided by Applied Biosystems. The cycle number at which the

7 ΔRn of a given reaction crossed the half height bar was denoted Ct. The Ct data from each sample were

8 normalized to the internal standard (RAc1) Ct using the formula (ΔCt=target Ct – internal standard Ct).

9 The ΔCt values of β were compared with the ΔCt value of α' using the formula (ΔΔCt=ΔCt (α') - ΔCt (β)).

10 Relative quantification (RQ) and relative gene expression were measured according to the formula

11 (RQ=2^{ΔΔCt}).

12 **Gel-filtration chromatography**

13 The molecular assembly of the α' and β subunits and modified α' subunit expressed in rice seeds

14 was analyzed by gel-filtration using a Hi-Prep 26/60 Sephacryl S-300 HR column, equilibrated with buffer

15 A (35 mM sodium phosphate (pH 7.6), 0.4 M NaCl, 1 mM EDTA, 0.02 % (w/v) NaN₃) containing 10 mM

16 ME at a flow rate of 0.5 ml/min.

17 **Endo H and PNGase F digestion**

1 Five seeds from transgenic rice expressing α' or β subunit were ground with a mortar and pestle,
2 and proteins were extracted with denaturing buffer (0.5 % SDS, 1 % ME, 100mM Tris-HCl, pH 8.0).
3 Purified α' and β subunits from soybean were denatured with denaturing buffer. The final concentrations
4 of extracted proteins and purified α' and β subunits were adjusted to 0.25 mg/ml with denaturing buffer.
5 Samples were split into three tubes and incubated with 30 mU of endoglycosidase H (Endo H; Bio Labs)
6 and storage buffer of Endo H (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) as a control, or 30 mU of
7 peptide:N-glycanase F (PNGase F; Bio Labs) and storage buffer of PNGase F (20 mM Tris-HCl (pH 7.5),
8 50 mM NaCl, 5 mM EDTA, 50 % glycerol) as a control, at 37 °C for 3 hr. Total protein was then
9 precipitated adding 1 volume of 30 % trichloroacetic acid, and the protein pellet was washed twice with
10 ice-cold acetone and dissolved with 45 mM Tris-HCl (pH 6.8) containing 2 % (w/v) SDS, 30 % glycerol,
11 and 0.1 M ME). Samples were analyzed by SDS-PAGE followed by Western blotting.

12 **Sequential extraction**

13 Five seeds from each of the transgenic T₁ plants were ground with a mortar and pestle and
14 sequentially extracted with three types of solutions at seed/solvent ratio 1:10 (w/v), each extraction being
15 followed by centrifugation at 30,000 x g for 15 min. The following solutions were used in two separate
16 extraction series: buffer A without ME, eight extractions; 1% lactic acid, four extractions; SDS buffer (45
17 mM Tris-HCl, pH 6.8, containing 2 % SDS, 30 % glycerol and 0.1 M ME). The difference between the

1 first and the second extraction series is the presence or absence of 20 mM ME in buffer A.

2 **Electron microscope immunocytochemical localization**

3 Mature and developing rice seeds were cut into 1.5 to 2.0 mm sections and fixed for 5 h in 1.5 %
4 (v/v) glutaraldehyde solution at 4 °C. Tissue sections were washed three times with buffer B (50 mM
5 sodium phosphate, pH 7.2), dehydrated (the ethanol wash, starting with 50%, 60% to 98 %, 1 hr for each
6 wash) followed by 100 % propylene oxide at −20 °C (three washings, 1 hr for each). The mature seed
7 samples were placed in epoxy resin (Quetol-812)/propylene oxide 1:3 (v/v) for 2 days, resin/propylene
8 oxide 3:1 (v/v) for 2 days, and finally 100 % resin for 2 days. Polymerization was done at 45 °C for 1 day,
9 and at 60 °C for 2 days. The developing seed samples were placed into LR white overnight at 4 °C and
10 transferred to beam capsules (NISSHIN EM, TOKYO) filled with freshly prepared resin. The resin was
11 allowed to polymerize for 2 days under indirect UV light at 4 °C.

12 Ultrathin sections (60-80 nm) were obtained with a glass knife and placed onto
13 formal/carbon-coated grids. The sections were blocked with 5 % (w/v) BSA-PBS and then incubated for 1
14 hr at room temperature on a drop of anti- α' , anti- β , anti-globulin, anti-glutelin, anti-10kDa prolamin and
15 16kDa prolamin serum diluted 1/1000, 1/4000, 1/1000, 1/200, 1/200 1/10000 in 1 % (w/v) BSA-PBS,
16 respectively. The sections were washed six times for 5 min each on a drop of 1 % (w/v) BSA-PBS and then
17 incubated on a drop of goat anti-rabbit IgG conjugated to 15-nm or 5-nm gold particles (H+L, Auro Probe

1 EM, Amersham) diluted 1/25 in 1 % (w/v) BSA-PBS for 30min at room temperature. After washing with
2 PBS, sections were washed twice with distilled water. The sections were stained for 25 min with 2 % (w/v)
3 uranyl acetate followed by incubation with 80 mM lead nitrate for 25 min. The grids were examined and
4 photographed using an electron microscope (model H-700H, Hitachi, Tokyo).

5 Seeds of three independent plants were observed for all constructs and the data was similar
6 among three independent plants. Representative images were shown as a figure.

7 **Statistical analysis**

8 Student t-tests (two-tailed, unequal variance) were performed using MS Excel. The term
9 statistical difference is used to indicate differences for which $P < 0.05$.

10

11 **Results**

12 **Transgenic rice seeds expressing α' and β subunits**

13 β -Conglycinin α' and β cDNAs were driven by the rice glutelin *GluB-1* and *GluB-2* promoter
14 regions in pGTV-HPT, respectively (Fig.1). The resulting pGTV-HPT/ α' and pGTV-HPT/ β were
15 introduced into rice calli by *Agrobacterium tumefaciens*-mediated transformation. Eleven and 22
16 independent lines expressing α' and β , respectively, were regenerated. Total protein extracted from T₁
17 seeds of the transgenic plants was spotted onto nitrocellulose membranes and the accumulation levels of

1 the α' and β subunits were estimated immunologically (Fig. 2). The accumulation levels of the α' subunit
2 was higher than those of the β subunit and the average levels of the α' and β subunits were 3.9% and 2.0%,
3 respectively. The accumulation level between them was statistically difference. The lines with the highest
4 accumulation levels of the α' and β subunits were subjected to subsequent analysis.

5 **Comparison of transcription levels of α' and β subunits in rice**

6 A difference in the transcription levels or translational efficiencies of α' and β mRNAs might be a
7 reason for the difference in accumulation levels of the α' and β subunits (Fig. 2). To verify this possibility,
8 we compared the quantities of both mRNAs by real-time PCR. The top lines having a single copy were
9 self-pollinated to obtain homozygous lines. The accumulation levels of the α' and β subunits in
10 homozygous lines were 7.9 ± 0.7 and $4.4 \pm 0.8\%$ in total proteins, respectively (data not shown). In the
11 homozygous line seeds, the α' subunit accumulated about twice as much as the β subunit, similarly with
12 the T1 seeds. The total RNAs from 15 days-after-flowering (15 daf) seeds of α' 6-2 and β 5-4 homozygous
13 lines were subjected to real-time PCR. Ct values of α' 6-2 and β 5-4 collected from half height bar crossed
14 each Δ RN were 18.9 and 18.5, respectively (Table 1). To correct the experimental error between the α' and
15 β subunits, we utilized the rice actin gene RAc1 as the internal standard. The Ct values of RAc1 in
16 transgenic rice seeds expressing the α' and β subunits were 28.5 and 28.1, respectively. To compare their
17 transcription levels, the Ct values were normalized. The results indicate that the transcription level of

1 α' 6-2 was close to that of β 5-4, although the accumulation level of α' 6-2 was about twice as much as that
2 of β 5-4. Thus, the transcription level was not reason for the difference in the accumulation levels of both
3 proteins in rice seeds. Thus, transcription levels do not explain the difference in the accumulation of the
4 two proteins in rice seeds. Translational efficiencies or protein stability may therefore account for the
5 difference in the accumulation level between the α' and β subunit.

6 **Post-translational modification of α' and β subunits in rice seeds**

7 The bands for the α' and β subunits were clearly detectable when total seed proteins from
8 transgenic rice were subjected to SDS-PAGE followed by western blotting (Fig. 3). No degradation
9 products of both the α' and β subunits were found by western blotting. Thus, both the α' and β subunits
10 stably accumulated in the rice seeds. The α' and β subunits extracted from transgenic rice seeds were
11 eluted at the same positions (100 min and 124 min, respectively) as α' and β homo-trimers purified from
12 soybean seeds on gel filtration chromatography (Fig. 4). This indicates that both the α' and β subunits from
13 rice seeds form trimers.

14 Both the α' and β subunits are N-glycosylated with high-mannose type glycans in soybean
15 (Yamauchi and Yamagishi, 1979). To examine whether the α' and β subunits synthesized in rice seeds were
16 N-glycosylated, they were subjected to digestion either by PNGase F (hydrolyzes almost all N-glycans,
17 excluding the core-fucosylated complex N-glycan) or by Endo H (primarily hydrolyzes high-mannose

glycan but not complex glycan). As a result of the digestion by PNGase F as well as by Endo H, both the α' and β subunits gave a single band of molecular mass lower than that of the intact subunit (Fig. 5). Thus, both the α' and β subunits were glycosylated with high-mannose N-glycan, and not with complex glycan, indicating that the N-glycan modification of the α' and β subunits in rice is similar to those in soybean seeds (Yamauchi and Yamagishi, 1979). Complex glycan modification of the α' and β subunits does not occur in soybean and rice seeds, although most of them traffic through the Golgi apparatus indicating limit access to modifying enzymes to these proteins in developing seeds.

Interaction of α' and β subunits with rice seed storage proteins

To examine whether the α' subunit containing Cys13 in the mature subunit interacts with glutelin in transgenic rice seeds via a disulfide bridge, we conducted sequential extractions of the seeds with buffer A without ME, lactic acid and SDS buffer. The same extractions were done with transgenic rice seeds expressing the β subunit. The extracts were subjected to SDS-PAGE in the absence of ME followed by western blotting (Fig. 6A-I, II, III). Most (84 %) of the β subunit detected as a single band was extracted in fractions 1 and 2 (Fig. 6A-III). In contrast, a large amount of the α' subunit (39 %) was extracted with lactic acid in fraction 9 (Fig. 6A-I). When the extracts were subjected to SDS-PAGE in the presence of ME followed by western blotting, the α' subunit in fraction 9 as well as in fraction 1 showed a single band corresponding to the α' monomer (Fig. 6A-II). These results suggest that a part of mature α' subunit is

1 linked with rice acid-soluble proteins (mainly glutelin) via a disulfide bond.

2 To further characterize the interaction of rice proteins with the α' subunit, we subjected fraction 9
3 to two-dimensional (without/with ME) SDS-PAGE (Fig. 6B) followed by western blotting in the second
4 dimension using anti-glutelin (Fig. 6B-III) and anti- α' (Fig. 6B-IV) sera. Most of the coomassie-stained
5 proteins (first dimension) were identified in the second dimension as glutelin acidic chains (Fig. 6B-III).
6 One of the major bands of the α' subunit found in the second dimension migrated as α' monomer (Fig.
7 6B-IV; asterisk). Another major bands of the α' subunit might correspond to the complex of the α' subunit
8 and rice acid-soluble proteins (Fig. 6B-IV; double asterisk). Other weak bands of the α' subunit were also
9 detected in the high-molecular mass region (Fig. 6B-IV). Glutelins were detected in a region of molecular
10 mass higher than the α' monomer, suggesting that a part of the α' subunit forms one or more disulfide
11 bonds with glutelin.

12 In another experiment, we used a similar scheme of thirteen sequential extractions but added ME
13 in buffer A for extractions from fractions 5 to 8 (Fig. 7). The extracts were subjected to SDS-PAGE in the
14 presence of ME followed by western blotting. As expected, most of the β subunit was found in the first two
15 fractions. However, only about half (51 %) of the α' subunit extracted by buffer A without ME
16 corresponded to the α' trimers (Fig. 6 A-II). Most of the rest of the α' subunit (41 %) was extractable only
17 by buffer A with ME. These results together with Fig. 6A-II indicate that the α' subunit is linked with

1 glutelin by one or more disulfide bonds.

2 **Transgenic rice seeds expressing mutated α' subunit**

3 To study the role of Cys residues of the α' subunit (four residues in the pro-region and Cys13 in
4 the mature subunit) in the accumulation behavior of the α' subunit in rice seeds, cDNAs for the deletion
5 mutants driven by the *GluB-1* promoter were introduced into rice calli ($\alpha'\Delta$ Cys1 and $\alpha'\Delta$ Cys5; see Fig.
6 1A). The pro region of $\alpha'\Delta$ Cys1 is supposed to be removed in the vacuoles. The average accumulation
7 levels of $\alpha'\Delta$ Cys1 and $\alpha'\Delta$ Cys5 in total rice seed proteins were 3.2 and 2.5%, respectively (Fig. 2). A
8 statistical difference of the accumulation level between α' and $\alpha'\Delta$ Cys5 could not be found. This indicates
9 that the higher accumulation of the α' subunit with respect to the β subunit might not be due to interactions
10 with glutelin by a disulfide bond. Both the substitution of Cys13 and the deletion of the pro region did not
11 affect the self-assembly of $\alpha'\Delta$ Cys1 and $\alpha'\Delta$ Cys5 (Fig. 4B). Similar to the β subunit, more than 90 % of
12 the total $\alpha'\Delta$ Cys1 and $\alpha'\Delta$ Cys5 from transgenic rice seeds were extractable with buffer A without ME (Fig.
13 7).

14 **Subcellular localization of β -conglycinins in transgenic mature seeds**

15 The subcellular localization of the α' and β subunits, $\alpha'\Delta$ Cys1 and $\alpha'\Delta$ Cys5 in transgenic rice
16 seeds was studied by immunocytochemical analysis (Fig. 8). The electron density of PB-II was uniform in
17 mature non-transgenic seeds (Fig. 8A). In contrast, low electron density regions at the periphery of PB-II

were detected in mature seeds of transgenic rice expressing the β subunit. The β subunit localized in low electron density regions (Fig. 8C). Remarkably, in the case of mature seeds of transgenic rice expressing the α' subunit, the electron density of the entire PB-II was high, although the α' subunit was detected only in the peripheral region (Fig. 8B). On the other hand, low-density regions were formed in PB-II in mature seeds of transgenic rice expressing $\alpha'\Delta\text{Cys5}$ and $\alpha'\Delta\text{Cys1}$ (Fig. 8D-F). $\alpha'\Delta\text{Cys5}$ was localized only in the low-density regions (Fig. 8D), whereas $\alpha'\Delta\text{Cys1}$ was found in both low- and high-density regions (Fig. 8E and F). These results indicate that the pro region of the α' subunit plays a fundamental role in the localization within PB-II.

9 Subcellular localization and trafficking of β -conglycinin in developing seeds

To investigate the trafficking of the α' subunit in rice seeds, developing seeds (10 days after flowering, 10 daf) of transgenic rice expressing the α' subunit were analyzed by electron microscopy (Fig. 9). In the early stage of PB-II formation, the α' subunit localized in the peripheral regions of PB-II (Fig. 9A). Low electron density regions of PB-II were not observed in immature seeds of transgenic rice expressing the α' subunit similar to mature seeds of transgenic rice expressing the α' subunit. Vesicles possibly budding from the Golgi apparatus were labelled by gold particles against the α' subunit antibody (Fig. 9B). Glutelin was also transported to PB-II by vesicles through the Golgi apparatus (Fig. 9C) as reported previously (Krishnan et al., 1986). Moreover, glutelin was observed in regions where the α'

1 subunit accumulated (Fig. 9D). This observation is consistent with the result that the α' subunit interacted
2 with glutelin within rice seeds by a disulfide bond (Fig. 6).

3 In the developing seeds of transgenic rice expressing the β subunit, some PB-II had high and low
4 electron density regions in analogy with mature seeds of transgenic rice expressing the β subunit. The gold
5 particles against anti- β serum existed primarily in the low electron density regions of PB-II (Fig. 10A). On
6 the other hand, gold particles against anti-glutelin serum were not observed in the low electron density
7 regions of PB-II (Fig. 10B). Further, the β subunit was also observed in dense vesicles (Fig. 10C) and in
8 morphologically different compartments (Fig. 10D). They were surrounded by ribosomes just like
9 precursor accumulating (PAC) vesicles which were reported to carry storage proteins from the ER to the
10 vacuoles bypassing the Golgi apparatus (Hara-Nishimura et al., 1998). Binding protein (BiP), a chaperon
11 located in the ER, was observed in PAC-like vesicles (Fig. 10E). Further, electron-dense aggregate was
12 also observed in the ER lumen, and prolamin could be barely observed in this aggregate (Fig. 10F). These
13 results indicate that the PAC-like vesicle was different from PB-I, although it was derived from the ER in
14 analogy with PB-I. This suggests that the PAC-like vesicle transported a part of the β subunit from the ER
15 to the vacuole.

16 Developing seeds of transgenic rice expressing $\alpha'\Delta\text{Cys5}$ had two electron density regions in
17 PB-II in analogy with transgenic rice seeds expressing the β subunit. $\alpha'\Delta\text{Cys5}$ existed mainly in the low

1 electron density region (Fig. 11A). On the other hand, glutelin accumulated in high electron density
2 regions, but not in low electron density regions (Fig. 11B). Thus, α' Δ Cys5 and glutelin also separately
3 accumulated in PB-II. These phenomena are very similar to those of the β subunit in rice seeds of
4 transgenic rice expressing the β subunit. Further, α' Δ Cys5 was also observed in the Golgi apparatus and
5 the dense vesicles (Fig. 11C). However, PAC-like vesicles were not observed in transgenic rice seeds
6 expressing α' Δ Cys5, although low electron density regions were observed in PB-II.

7 α' Δ Cys1 accumulated in both high and low electron density regions in PB-II in developing seeds
8 (Fig. 12A, B) and the PAC-like vesicles were not observed. In low electron density regions, glutelin was
9 not observed in analogy with the case of β subunit and α' Δ Cys5 (data not shown). Moreover, glutelin was
10 also observed in high electron density regions where α' Δ Cys1 was localized (Fig. 12C). These results with
11 sequential extraction experiment suggest that the α' subunit interacts with glutelin via the pro region in rice
12 seeds and that a disulfide bond plays an important role on the interaction.

13

14 Discussion

15 Complex formation of the α' subunit and glutelin in transgenic rice seeds

16 In this study, we introduced α' and β cDNAs of soybean β -conglycinin driven by glutelin promoters
17 into the rice genome to investigate their accumulation behavior. Transcription levels of the α' and β

1 cDNAs in developing seeds of transgenic rice were found to be similar to each other (Table 1). Further,
2 both α' and β subunits were shown to undergo posttranslational modification in rice seeds similar to those
3 in soybean seeds (N-glycosylation by high-mannose glycans and detachment of N-terminal pro region
4 from the α' precursor) (Fig. 5). The accumulation level of the α' subunit in mature rice seeds was about
5 two-times higher than that of the β subunit (Fig. 2). The α' subunit and glutelin co-localized in high
6 electron density regions in PB-II in developing seeds (Fig. 9), whereas the β subunit localized only in a
7 low electron density region (Fig. 10). A sequential extraction experiment showed that the α' subunit could
8 form a disulfide bond with glutelin (Fig. 6). Further, we examined the behavior of two kinds of modified
9 α' subunit ($\alpha'\Delta$ Cys5 devoid of all Cys by means of removal of its pro region and substitution of Cys13
10 with serine, and $\alpha'\Delta$ Cys1 containing intact pro region and substituted Cys13) in transgenic rice seeds to
11 elucidate the role of Cys residues in the accumulation of the α' subunit. Both $\alpha'\Delta$ Cys5 and $\alpha'\Delta$ Cys1 ,
12 similar to α' subunit, formed trimers (Fig. 4). The $\alpha'\Delta$ Cys5 localized in low-density regions in PB-II
13 similarly to the β subunit, whereas $\alpha'\Delta$ Cys1 localized in low- and high-density regions (Fig. 8). These
14 results suggest that the α' subunit makes a complex with glutelin via one or more disulfide bonds in rice
15 seeds. Previous reports suggest that there could be a “dominant” effect of the storage proteins in directing
16 foreign proteins to the vacuole in seed cells (Arcalis et al. 2004; Drakakaki et al. 2006). Stabilization by
17 disulfide bonds might contribute a tendency for heterotypic interactions of storage proteins.

1

2 **Trafficking of β -conglycinin in rice seed**

3 Although PAC-like vesicles were observed in late developing stage (15 daf) of non-transgenic
4 rice seeds (Takahashi et al., 2005), in analogy with pumpkin seeds (Hara-Nishimura et al., 1998), they
5 were not observed in the nontransgenic rice of this study (10 daf). This suggests that the traffic ability of
6 early developing rice seed is sufficient to transport storage proteins from the ER to the vacuoles through
7 the Golgi apparatus. However, PAC-like vesicles were observed in the early developing stage (10 daf) of
8 transgenic rice seeds expressing the β subunit, although the accumulation level of the β subunit was lower
9 than that of the α 'subunit. This suggests that introduction of the β gene in the rice induced the PAC-like
10 vesicle formation in the early developing stage.

11 There have been reports on the direct pathway from the ER to the vacuole, introduced by
12 transgenes. Introduction of a gene of sulfhydryl-endopeptidase (SH-EP) which has a KDEL-tail,
13 papain-type vacuolar proteinase of germinated *Vigna mungo* seeds in *Arabidopsis* resulted in the formation
14 of KDEL-vesicle, transported to the vacuoles by a Golgi-independent. Such vesicle was not observed in
15 *Arabidopsis* seeds expressing SH-EP mutant lacking the KDEL-tail and nontransgenic *Arabidopsis* seeds
16 (Okamoto et al., 2003). When a gene for KDEL-tagged β -phaseolin, 7S storage protein of French bean
17 (*Phaseolus vulgaris*), was introduced into tobacco leaf protoplasts, KDEL-tagged β -phaseolin was

1 transported to the vacuole but the complex glycan was not formed, although normal β -phaseolin had the
2 complex glycan. These suggest that the KDEL-tagged β -phaseolin was transported to the vacuoles directly
3 from the ER (Frigerio et al., 2001). When human serum albumin containing N-terminal signal sequence
4 and C-terminal KDEL tag was introduced into wheat and expressed in seeds, it was also transported to the
5 vacuoles through the Golgi-independent route (Arcalis et al., 2004). It is likely that the overexpression of
6 transgenes contained the KDEL-tail coding sequence in the ER caused ER stress, and that the ER stress
7 induced the expression of some kind of molecular chaperons or transmembrane proteins resulting in a
8 Golgi-independent pathway. The β subunit does not contain the KDEL sequence, but a direct transport
9 pathway from the ER to the vacuoles was induced. In soybean seeds, ER-derived protein bodies (PBs)
10 were observed at high frequency in the mutant line containing glycinin composed of only group I subunits
11 the solubility of which was lower than that of the normal glycinin (Mori et al., 2004). Consequently, there
12 is a possibility that the induction of PAC-like vesicle in rice seeds also depends on physicochemical
13 properties, such as solubility and surface hydrophobicity, of the β subunit. The pH in the ER is estimated to
14 be approximately 7.1 in resting HeLa cells (Kim et al., 1998). The α' subunit is soluble at $\mu=0.08$ and pH
15 7.1, whereas the β subunit is insoluble under these conditions (Maruyama et al., 1998, Maruyama et al.,
16 2002). These findings suggest that the β subunit is insoluble in the ER lumen environment of rice seed and
17 partly aggregates. In contrast, the α' subunit, $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$, containing the hydrophilic domain

(extension domain), are soluble in the ER lumen environment, so all of them could be transported to the vacuoles via the Golgi apparatus. Recently, we showed that aggregated-type of red fluorescent protein forms ER-derived compartment in soybean and *Arabidopsis* seeds (Maruyama et al., 2008). Application of aggregated-type of red fluorescent protein could further elucidate a formation of ER-derived compartment in rice seeds.

Development of highly-physiologically functional rice to improve human health

β -Conglycinin has many physiological functions. It has been reported that the α' subunit decreases plasma cholesterol and triglyceride levels of rat when rats were fed 20 mg (kg body weight·day) of the α' subunit (Duranti et al., 2004). Thus, 1.2 g (60kg body weight·day) of α' subunit are needed for possible effective physiological functions in human. The maximum accumulation level of the α' subunit in total rice seed proteins was about 8%, and rice seed proteins account for 7% of total dry weight of rice seed. If one considers that average daily consumption of rice in Japan is 150g, which would contain about 0.84 g of α' , increasing the accumulation levels of α' subunit by a factor of 1.5 is necessary to confer physiological functions to rice seeds.

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6

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- 3

1 Figure Legends

2 **Figure 1.** Schematic presentation of the structure of wild-type and mutated β -conglycinin subunits (A) and
3 fusion genes used for rice transformation (B). A; wild-type α' and β subunit and α' modified versions
4 $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$. Positions of Cys residues (SH) are shown. B, the α' , β , $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$
5 fusion genes. *GluB-1*, glutelin B-1 gene; *g7*, gene 7; HPT, hygromycin phosphotransferase.

6
7 **Figure 2.** Comparison of the accumulation levels of α' and β subunits, $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$ in
8 respective transgenic rice seed determined by dot immunoblotting. Total protein was extracted from each
9 of the transgenic seeds with SDS buffer. Aliquots (1 $\mu\text{g}/1 \mu\text{l}$) were spotted on a nitrocellulose membrane
10 and the recombinant proteins were detected immunologically with either anti- α' or anti- β sera.
11 Accumulation levels of recombinant proteins were expressed as a percentage of respective total seed
12 protein. Each mark represents the accumulation level in an independent transgenic plant.

13
14 **Figure 3.** Detection of α' and β subunits from transgenic rice seeds by SDS-PAGE and western blotting.
15 Total seed protein extracted with SDS buffer was subjected to SDS-PAGE (lanes 1-3, CBB staining) and
16 western blotting (lanes 4, 5). Lane M, molecular mass markers; lane 1, non-transgenic seeds; lanes 2 and 4,
17 transgenic seeds expressing α' subunit; lane 3 and 5, transgenic seeds expressing β subunit. An arrow

1 indicates position of glutelin precursor.

2

3 **Figure 4.** Molecular assembly of recombinant proteins analyzed by gel filtration followed by western

4 blotting. A. Purified α' and β homo-trimers from soybean seeds and bovine serum albumin (66 kDa) used

5 as molecular mass markers were subjected to gel filtration on Sephacryl S-300 HR column and detected by

6 A₂₈₀. B, α' and β subunits and $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$ were extracted from transgenic rice seeds with

7 buffer A containing ME and subjected to gel filtration using the same column as in A. Fractions collected

8 every four minute were subjected to SDS-PAGE followed by Western blotting.

9

10 **Figure 5.** Analysis of N-glycosylation of α' and β subunits extracted from transgenic rice seeds. α' and β

11 subunits extracted from rice seeds were incubated in the absence (-) or presence (+) of either Endo H or

12 PNGase F at 37°C for 3 h. Reaction mixtures were subjected to SDS-PAGE followed by western blotting.

13

14 **Figure 6.** Sequential extraction of α' and β subunits from transgenic rice seeds. A. Seeds were treated with

15 buffer A containing no ME (eight extractions, lanes 1-8), then with 1 % lactic acid (four extractions, lanes

16 9-12), and finally with SDS buffer (lane 13). Each extract (fraction) was subjected to SDS-PAGE in the

17 absence (I) or presence (II, III) of ME followed by western blotting with anti- α' (I, II) and anti- β (III).

1 Single and double asterisks indicate positions of α' monomer and dimer, respectively. B, Two-dimensional
2 SDS-PAGE and western blot analysis of the fraction 9 of Fig. 6A-II. Arrow and arrow box indicate
3 directions of electrophoresis in first (-ME) and second (+ME) dimensions, respectively. First dimension:
4 CBB stained molecular mass markers (M), α' subunit purified from soybean seeds (I) and the fraction 9
5 (II). Second dimension, western blot analysis of fraction 9: immunoreactions with anti-glutelin (III) and
6 anti- α' (IV) sera. Closed and open arrowheads indicate the position of glutelin precursor and glutelin
7 acidic polypeptides, respectively.

8
9 **Figure 7.** Sequential extraction of recombinant α' and β subunit and α' Δ Cys1 and α' Δ Cys5 from
10 respective transgenic rice seeds. Seeds were treated with buffer A containing no ME (four extractions,
11 lanes 1-4), then with buffer A containing ME (four extractions, lanes 5-8), with 1 % lactic acid (four
12 extractions, lanes 9-12), and finally with SDS buffer (lane 13). Each extract (fraction) was subjected to
13 SDS-PAGE in presence of ME followed by Western blotting using with anti- α' (α' , α' Δ Cys1 and
14 α' Δ Cys5) and anti- β (β) sera.

15
16 **Figure 8.** Electron micrographs of mature seeds of non-transgenic and transgenic rice. A, non-transgenic
17 seed non-treated with anti-serum. Immunoreactions were done with anti- β serum for transgenic seed

expressing the β subunit (C) and anti- α' serum for transgenic seeds expressing the α' subunit (B), $\alpha'\Delta\text{Cys5}$ (D), and $\alpha'\Delta\text{Cys1}$ (E, F). Gold particles, 15 nm; bars, 0.5 μm .

Figure 9. Electron micrographs of developing seeds (10 daf) of transgenic rice expressing the α' subunit.

Immunoreactions were done with anti- α' (A, B) and anti-glutelin (C). Double immunoreactions were done with anti- α' (5nm) and anti-glutelin (15nm) (D) sera. GA indicates Golgi apparatus. Arrowheads indicates the position of α' . Bars = 0.5 μm .

Figure 10. Electron micrographs of developing seeds (10 daf) of transgenic rice expressing the β subunit.

Immunoreactions were done with anti- β (A, C, D), with anti-glutelin (B), anti-BiP (E) and anti-prolamin (F) sera. Bars = 0.5 μm .

Figure 11. Electron micrographs of developing seeds (10 daf) of transgenic rice expressing $\alpha'\Delta\text{Cys5}$.

Immunoreactions were done with anti- α' (A and C) and anti-glutelin (B) sera. Bars = 0.5 μm .

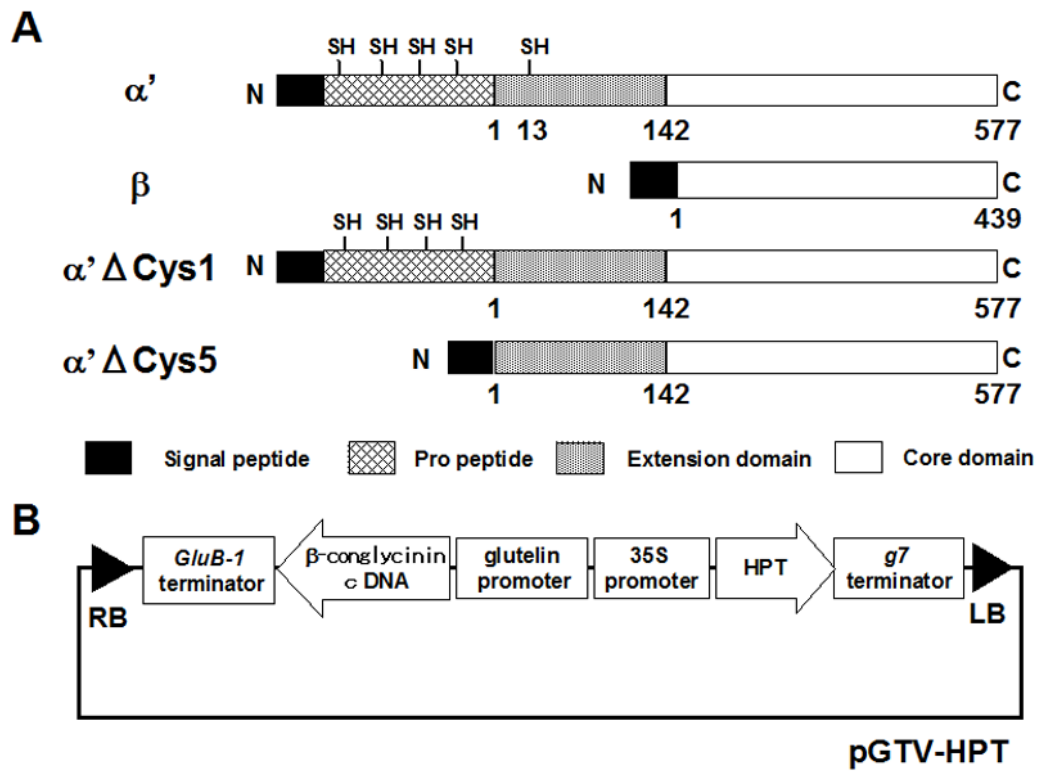
- 1 **Figure 12.** Electron micrographs of developing seeds (10 daf) of transgenic rice expressing α' Δ Cys1.
- 2 Immunoreactions were done with anti- α' sera (A, B). Double immunoreaction was done with anti- α'
- 3 (5nm) and anti-glutelin (15nm) (C) sera. Bars = 0.5 μ m.
- 4

1 **Table 1. Comparison of transcription levels of α' and β subunits in rice seeds.**

2

	Target Ct	Internal standard Ct (RAc1)	Δ Ct	$\Delta\Delta$ Ct	Relative quantification
α' 6-2	18.9	28.5	9.60	0	1
β 5-4	18.5	28.1	9.57	-0.03	0.98

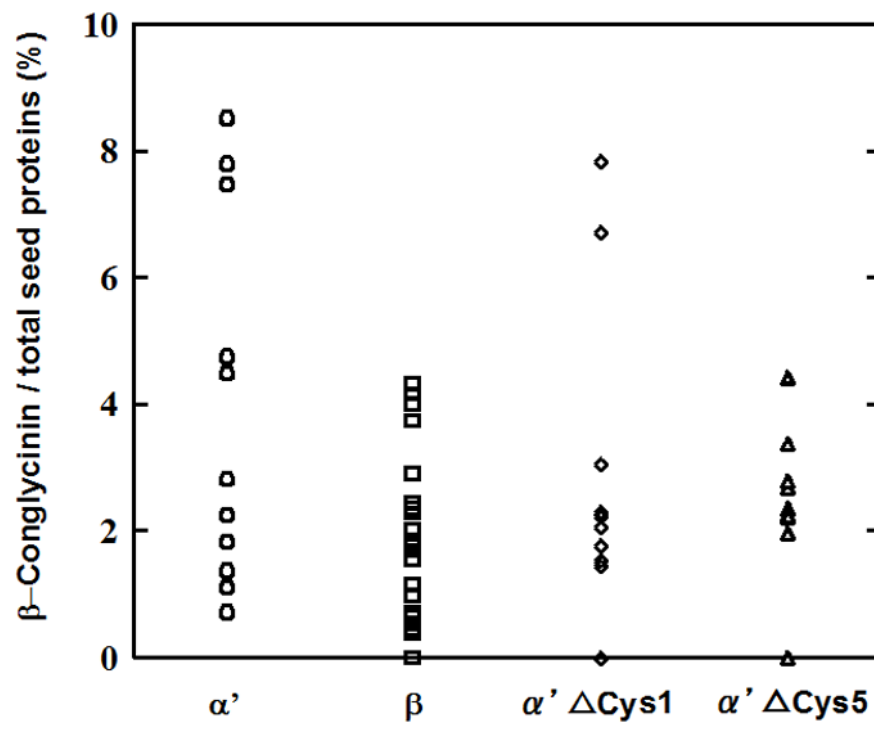
Fig. 1



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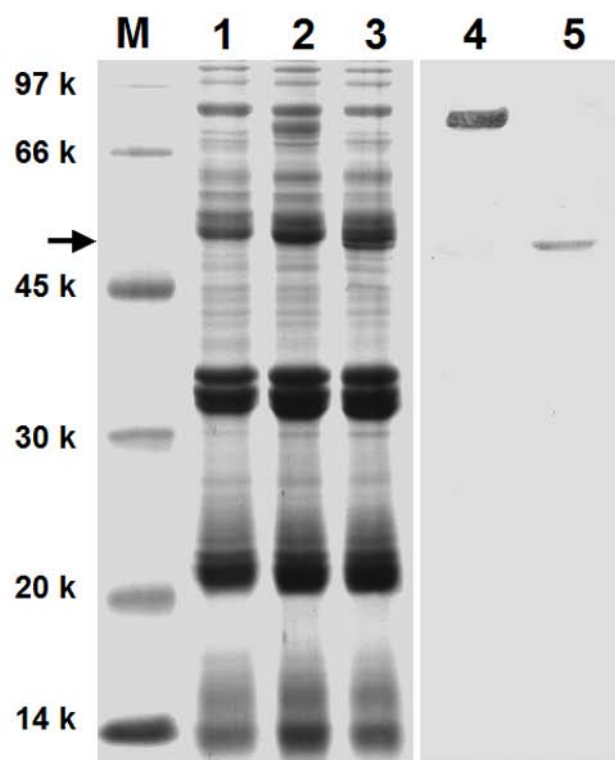
Fig. 2



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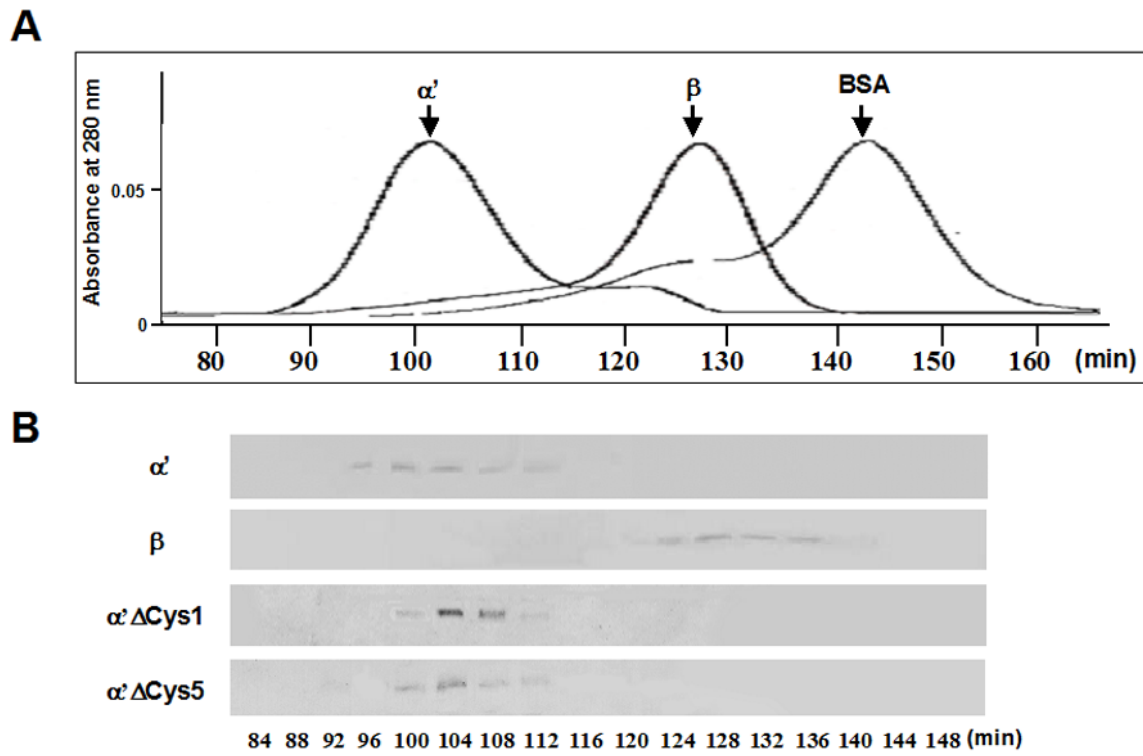
Fig. 3



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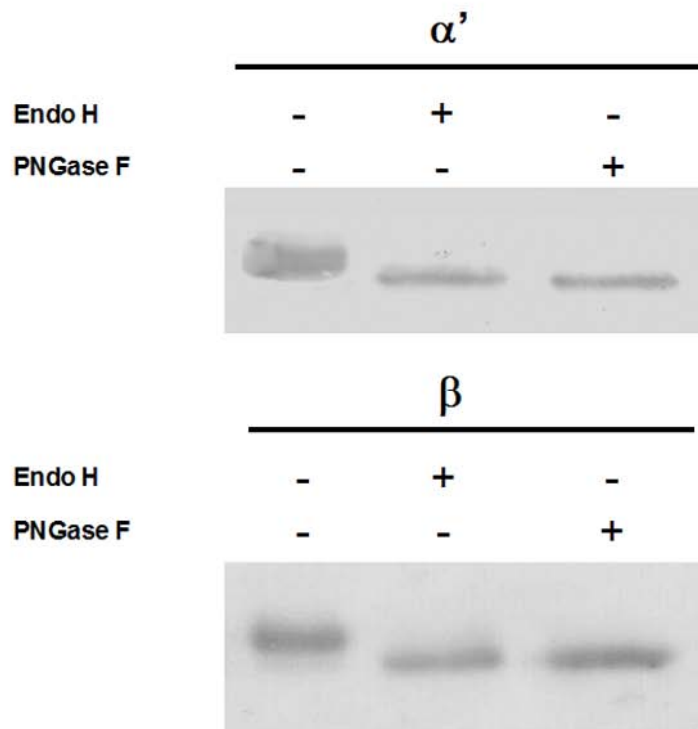
Fig. 4



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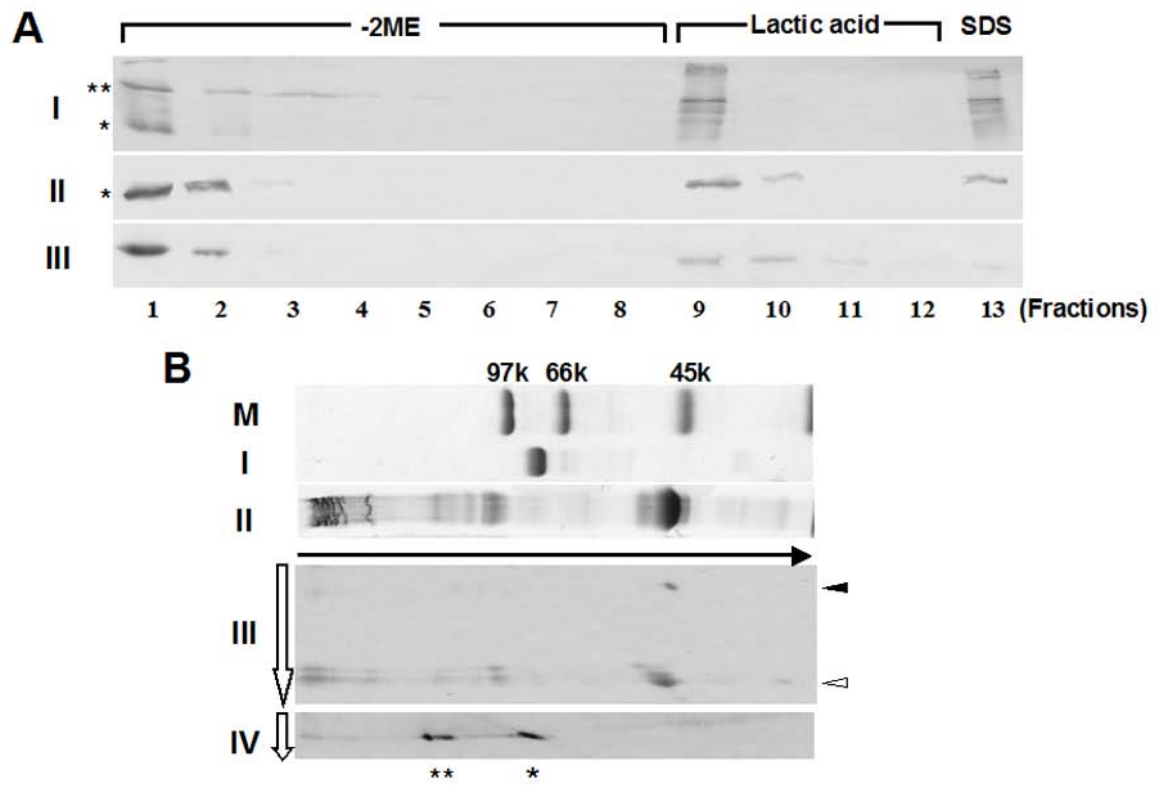
Fig. 5



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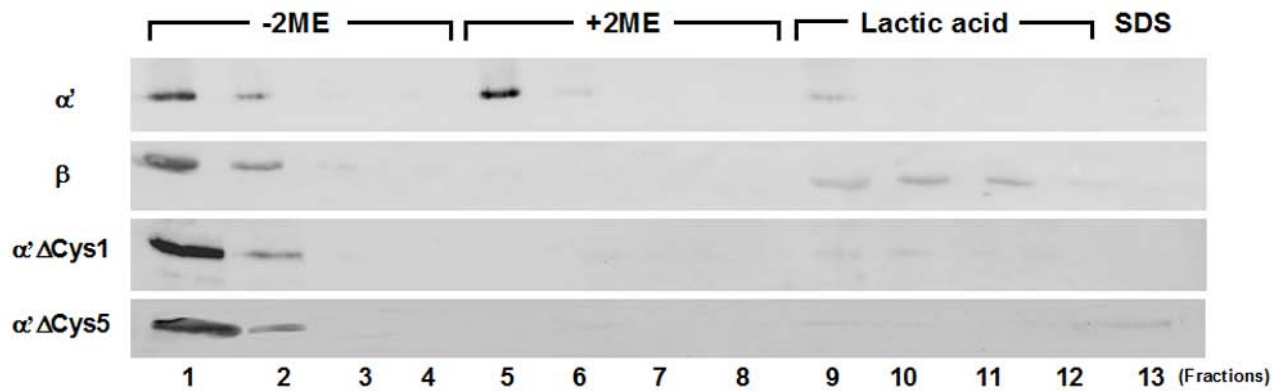
Fig. 6



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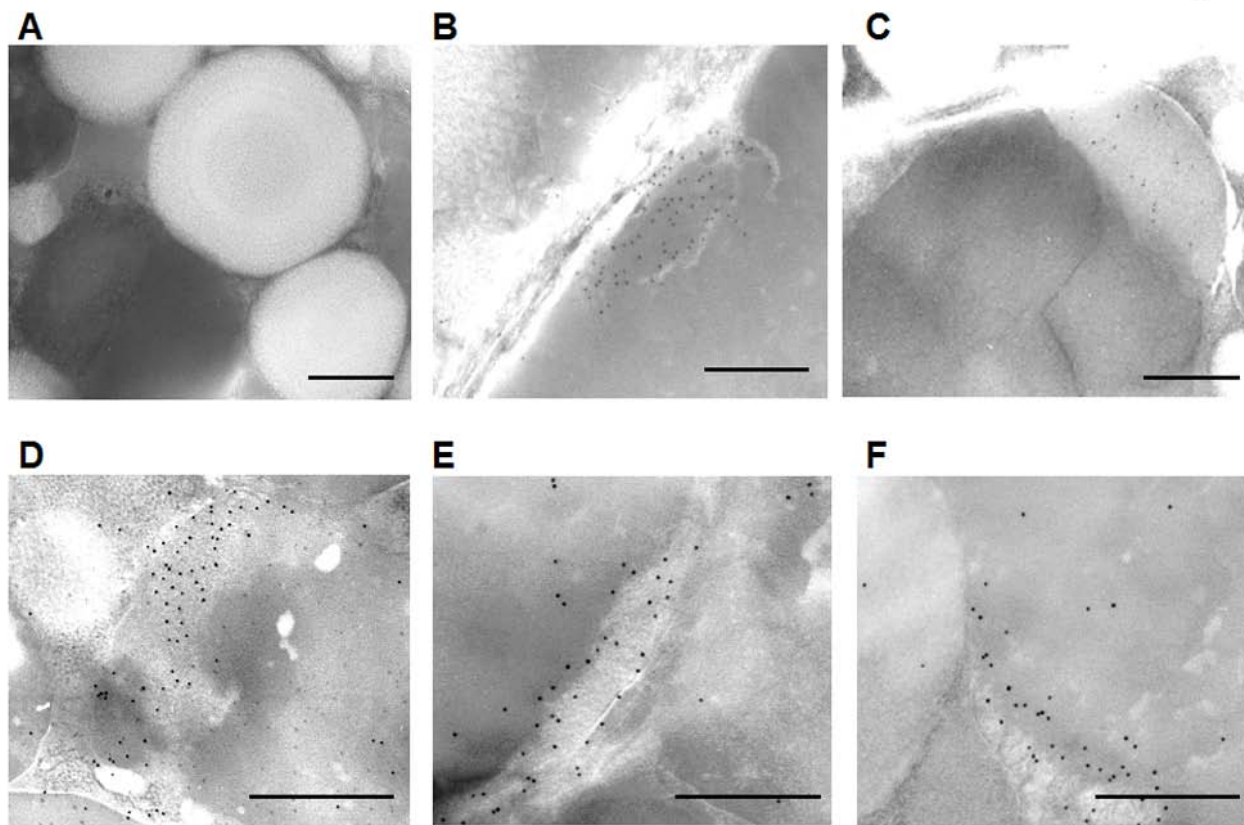
Fig. 7



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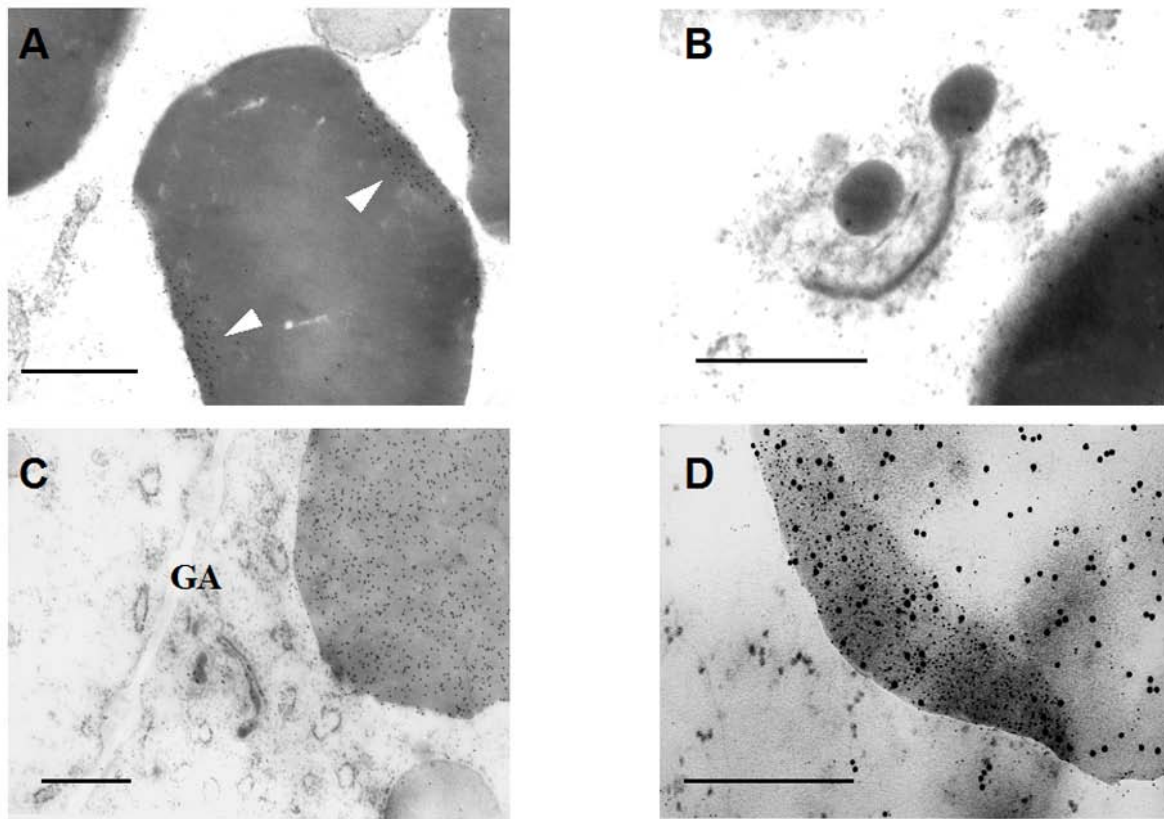
Fig. 8



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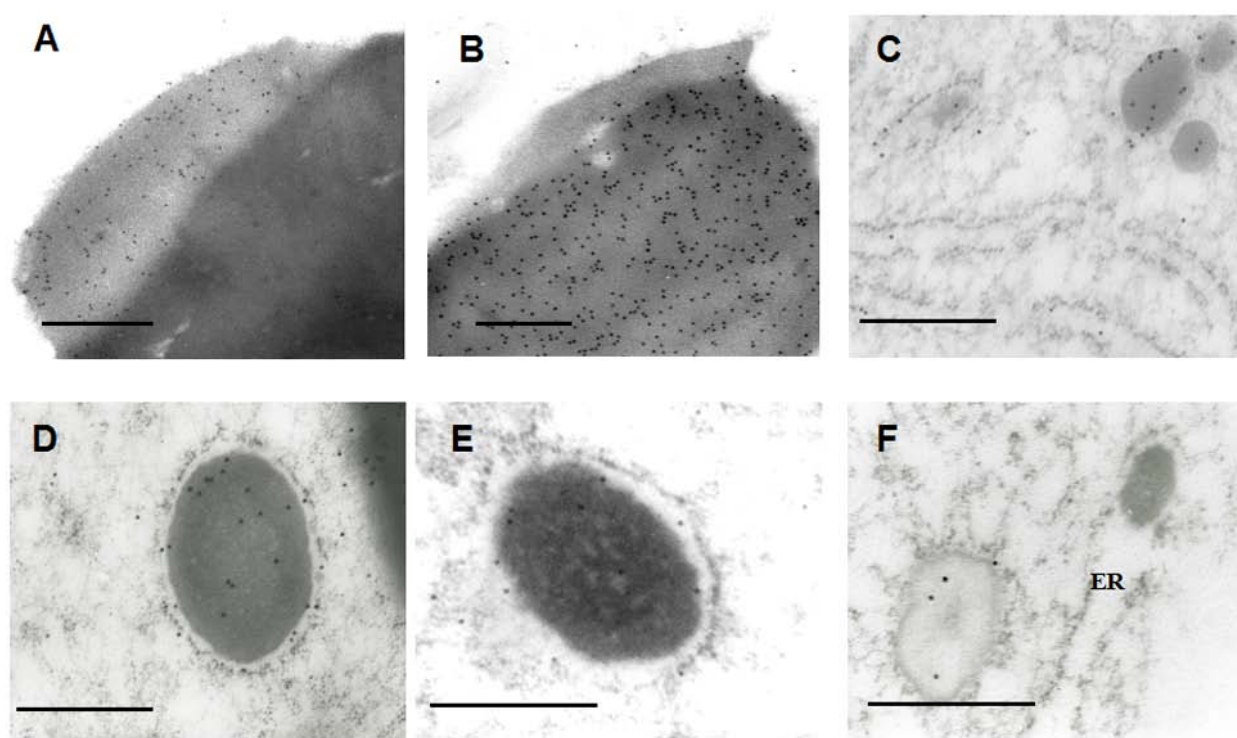
Fig. 9



1

2

Fig. 10



1

2

Fig. 11

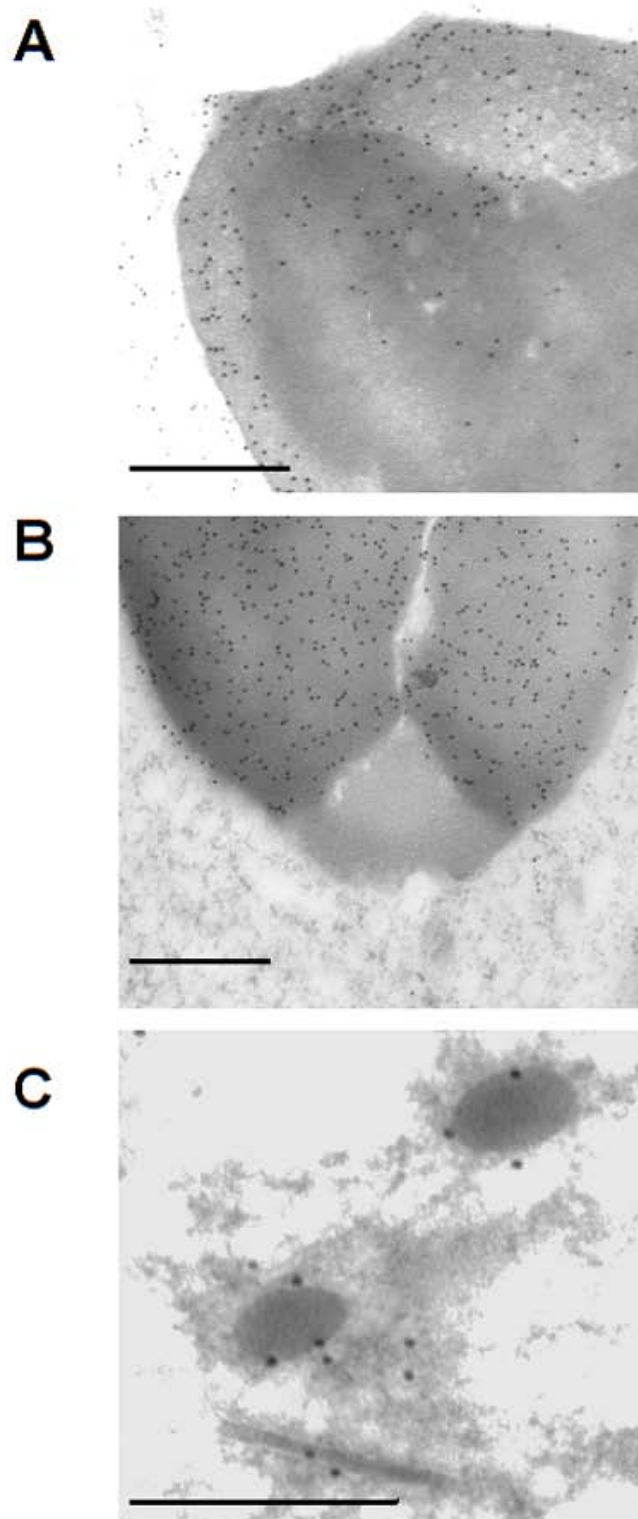


Fig. 12

